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Serological Correspondence Among Horseshoe "Crabs" (Limulidae)[1]

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(Plate I: Text-figures 1 & 2)

INTRODUCTION

THE FEW RESEARCHERS, Graham-Smith (1904), Boyd (1937), Wilhelmi (1942, 1944) and Leone & Webb (1952), that have reported on systematic aspects of the blood serum of horseshoe "crabs," have all compared *Limulus* serum with that of animals outside of the family. Since the present report is the first on the comparative serology of the Limulidae, it constitutes an introduction to the serological relationships among horseshoe "crabs." Three of the four extant species, representing the two subfamilies, were serologically compared: the North American species *Limulus polyphemus* (Linnaeus) Müller, Limulinae, and two Indo-Pacific species, *Carcinoscorpius rotundicauda* (Latreille) Pocock, and *Tachypleus gigas* (Müller) Leach, Tachypleinae. The fourth, the remaining Asiatic species, *Tachypleus tridentatus* Leach, was not studied.

The data, obtained by turbidimetric measurements and agar-diffusion records of the precipitin reaction between antigens and antibodies, were limited by the available supply of serum from the Indo-Pacific species.

Our serological findings provide a basis for a more comprehensive study on the systematics of the Limulidae. There is questionable support for the taxonomy proposed by Pocock (1902), who divided into three genera the four extant species previously confounded under *Limulus*.

MATERIALS AND METHODS

Hemolymph samples were collected from adult limuli in eleven populations of *Limulus*, from Florida to Maine, during the summer of 1953. Sera for this study were selected from animals in four widely separated populations: Pleasant Bay, Massachusetts; Providence River, Rhode Island; Miles River, Maryland; and Sarasota Bay, Florida. Sera from two of the three Indo-Pacific species, *C. rotundicauda* (samples from eleven specimens) and *T. gigas* (one sample), were obtained by Dr. D. S. Johnson and his associates in the Department of Zoology, University of Malaya. Without this generous assist from Dr. Johnson the present study would not have been possible; the author owes him a personal note of gratitude. Similar contacts with other researchers, to obtain *T. tridentatus* serum, proved nonproductive.

The hemolymph was preserved for study in the following manner (see Boyden, 1953, for general procedure). (1) A horseshoe "crab" was held in ventriflexion over a large glass beaker, or a funnel, and the arthrodial (dorsimesal) membrane in the hinge region cut deeply to puncture the underlying heart. Hemolymph usually flowed rapidly, directly from the heart; the flow could be increased by slowly moving the opisthosoma up and down like a bellows. (2) The hemolymph was stored overnight in an ice chest or a refrigerator to allow the amoebocytes to coagulate and form a "clot." (3) Then, after the clot formed, the serum was decanted into a graduate. (4) A stock solution of merthiolate (1 gram merthiolate powder in 100 milliliters of distilled water) was then added, 1 milliliter of the stock solution to each 49 milliliters of serum and the solution shaken well. (5) The merthio-

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lated serum was bottled and stored in a cold room until used (temperature regulated at 1°C.).

Antisera to the serum of female *Limulus* from six different populations and the Tachypleinae sera were produced, using rabbits as producers. A reciprocal testing program was followed, limited only by the small amount of Tachypleinae serum available. Full reaction curves for each of the antigens were run on the Libby photron-reflectometer (photron'er). In graphing the results, galvanometer readings on the photron'er were expressed as turbidity units along the ordinate, antigen concentration as antigen dilution along the abscissa. Each successive cell of antigen dilution, 1 through 15, contained one-half as much antigen as the preceding cell. The first cell, 1, was assumed to contain 5% antigen, or a titer of 1/20. Thus, cell 15 has an assumed antigen titer of 1/327,680. The exact protein concentration is not critical when whole titration curves are available for comparison. Total turbidity values for each curve were obtained by adding all the individual turbidities of each antigen dilution tube in the test. A value of 100 percent. was assigned to the area under the homologous curves and the heterologous values then expressed as percentages of the homologous total.

These serological tests were conducted at The Serological Museum, Bureau of Biological Research, Rutgers—The State University of New Jersey, under the direction of Drs. Ralph J. DeFalco and Douglas G. Gemeroy in 1954 and 1955. The techniques and procedures elaborated by Dr Alan A. Boyden, director of The Serological Museum, and co-workers (Boyden & DeFalco, 1943; Bolton, Leone & Boyden, 1948; and Leone, 1949, 1950) were used. The author is grateful to Dr. Boyden for reviewing the manuscript of this report.

Another measure of the precipitin reactions among Limulidae sera and antisera were obtained from a series of tests using the double-diffusion, Jennings-modified (Jennings & Malone, 1954; Jennings, 1954), three-depot method of Ouchterlony (1949). The desired test combinations of sera and antisera, each reactant placed separately in the corner reservoirs of a triangular plastic plate, separately diffuse through the agar. The diffusing reactants immediately combine in the region of equivalent concentrations (Munoz, 1954). At first invisible, this combination appears in time as a band or zone of precipitin which can be photographed. Dr. Sheldon A. London conducted these tests at the University of Delaware in 1957, in conjunction with a problem on microbial serological correspondence. The author is indebted to Dr. London for the photographs and the data he obtained.

Results

Reciprocal precipitin reactions between the sera and antisera of three species of Limulidae, *L. polyphemus, T. gigas* and *C. rotundicauda,* are summarized in Tables 1 and 2, and are shown on graphs (Text-fig. 1), a diagram (Text-fig. 2) and photographs (Plate I).

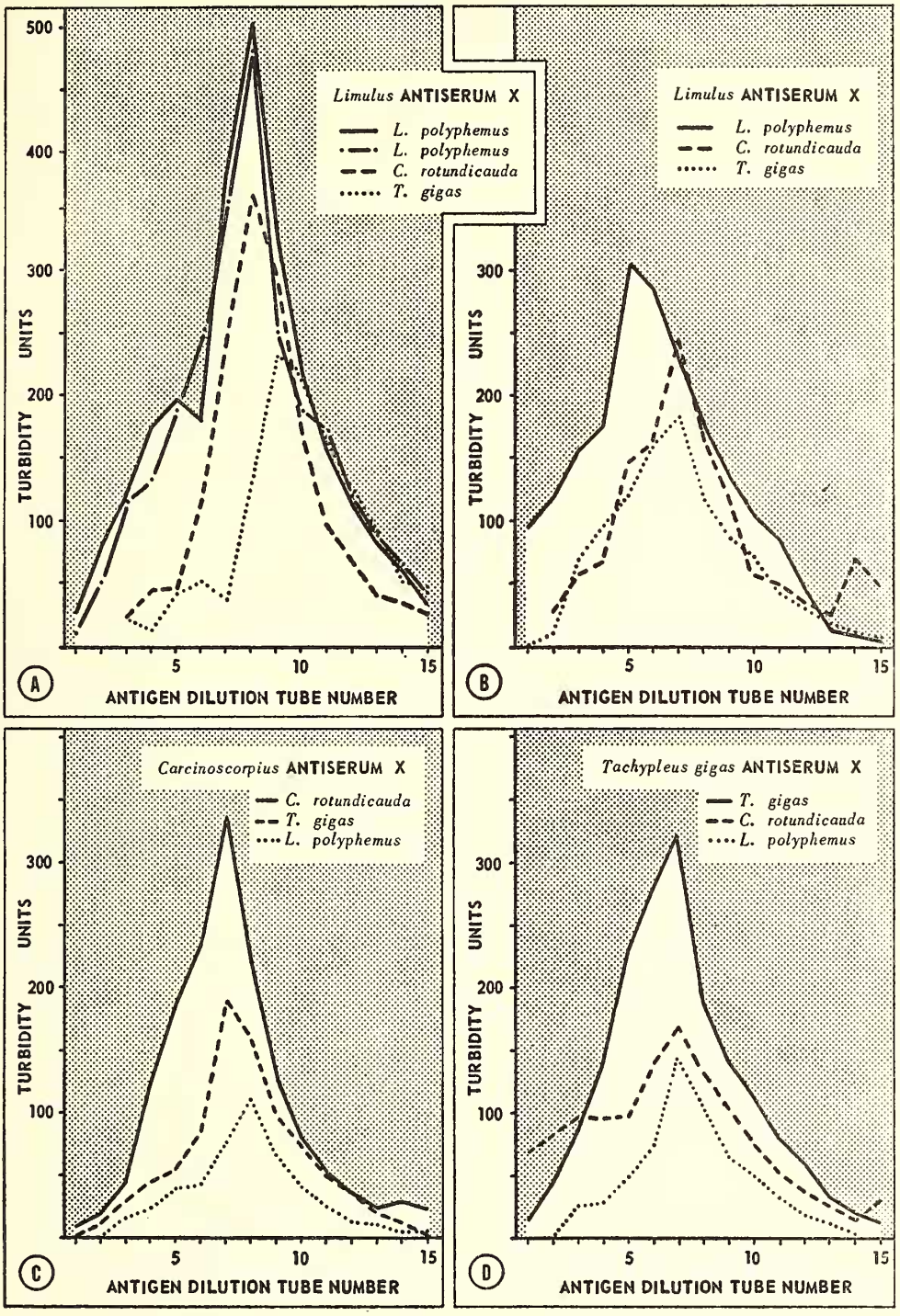
The curves (Text-fig. 1) obtained from the precipitin tests are typical unimodal curves which show no departure in spatial arrangement from a typical reaction curve (see for example: Leone, 1949; Boyden & DeFalco, 1956). The relative positions of the peaks of the curves are also typical, with the peaks of the heterologous curves usually to the right of and exceeded by the peak of the homologous reaction.

Turbidimetric results are summarized by a diagram (Text-fig. 2) indicating the serological distance between the species using a modification of Boyden's (1932) method. When the reaction values between two species are averaged and subtracted from 100, a serological "yardstick" is obtained which indicates the relative distances between the species. Thus the triangle in Text-figure 2B gives the relative distance between the three species according to a turbidimetric measurement of the precipitin reaction of their serum proteins in the reciprocal tests.

Reactions among sera and antisera for three *Limulus* from widely separated populations produce the typical homologous pattern of coalesced zones and form four precipitin bands in the agar plates (Plate I, Figures A, B). The crossed zones produced by tests between the three species show the unrelated character of at least two major components of the reactants (Plate I, Figures C, D) or one component (Plate I, Figures E, F). The *Tachypleus-Carcinoscorpius* reactants appear to produce the coalesced zones typical of strong serological correspondence (Plate I, Figures G, H). The asymmetry of the zones is ascribed to unequal concentrations of the reactants, with zone displacement toward the reservoir containing the reactant of lesser concentration.

Discussion

An examination of the turbidimetric data (Table 1 and Text-fig. 1) reveals the marked difference in the magnitude of the reaction of each of the antigens to the antisera and the shift to the right of the heterologous reaction curves in the region of antigen excess.



TEXT-FIG. 1. Precipitin titration curves showing the order of serological relationships among three species of horseshoe "crabs": *Limulus polyphemus, Carcinoscorpius rotundicauda* and *Tachypleus gigas*. The antisera and test sera (antigens) are given in the legend for each series of curves. The area under the homologous reaction curve is highlighted by the shaded background.

TABLE 1. RELATIVE AMOUNTS OF PRECIPITIN REACTION, RECORDED AS TURBIDITY IN PHOTRONREFLECTOMETER TESTS

These four sets of data (A-D) are represented graphically in Text-fig. 1.

Antiserum	Antigen	Peak Tube Number	Turbidity Units		
			Peak	Total	Percent. of Homologous Reactions
A. *L. polyphemus*	*L. polyphemus**	(8)	501	2641	100
	*L. polyphemus**	(8)	481	2502	95
	C. rotundicauda	(8)	361	1556	59
	T. gigas	(9)	230	1212	46
B. *L. polyphemus*	*L. polyphemus*	(5)	305	1924	100
	C. rotundicauda	(7)	247	1284	67
	T. gigas	(7)	183	1025	53
C. *C. rotundicauda*	*C. rotundicauda*	(7)	336	1549	100
	T. gigas	(7)	189	871	56
	L. polyphemus	(8)	112	477	31
D. *T. gigas*	*T. gigas*	(7)	320	1769	100
	C. rotundicauda	(7)	171	1208	68
	L. polyphemus	(7)	143	616	35

* Serum from specimens in two geographically different populations.

A comparison of all the reaction curves suggests that the several different rabbits employed in the production of the antisera reacted to the sera and produced comparable specificities of antibodies. The shift to the right of the heterologous curves, with respect to the homologous reaction, generally denotes a lesser amount of serological reaction between the heterologous sera and the test antisera. We are concerned here, however, with the relative placement of the species, from the homologous to the lesser reaction of the heterologous antigens, in each of the apparently linear serological series. These series are the basis for the two-dimensional diagram (Text-fig. 2) of the relationships among the three species. For a general discussion of the principles of systematic serology and the implications of the results of precipitin testing see Boyden (1942).

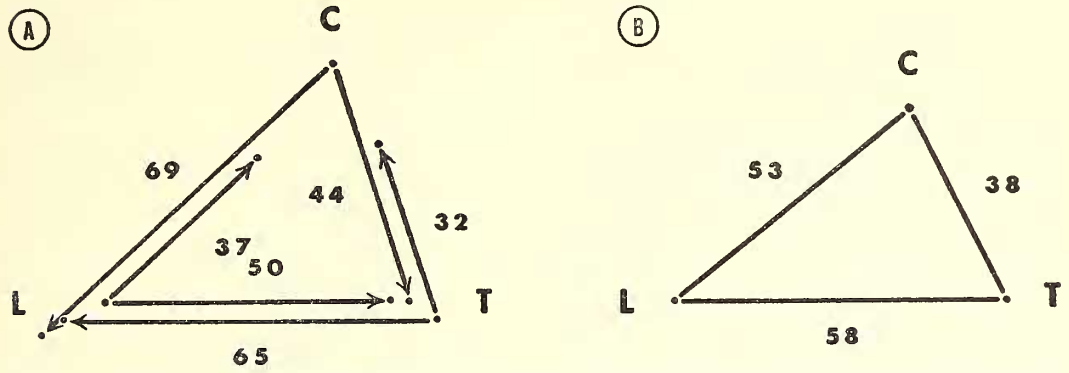
TABLE 2. SUMMARY OF THE TURBIDIMETRIC DATA ON THE RECIPROCAL PRECIPITIN REACTIONS, RECORDED AS PERCENT. OF THE HOMOLOGOUS REACTION. These data are graphically portrayed in Text-fig. 2.

Antiserum	Antigen		
	Limulus	*Carcinoscorpius*	*Tachypleus*
L. polyphemus	100	59	46
	100	67	53
C. rotundicauda	31	100	56
T. gigas	35	68	100

The serological placement of *L. polyphemus* slightly closer to *C. rotundicauda* than to *T. gigas* (Table 2 and Text-fig. 2B) requires further testing. Evidence (Shuster, 1958) indicates that *L. polyphemus* has a greater correspondence in morphometric and morphological characters with the genus *Tachypleus* than with *Carcinoscorpius*. Data for a definitive systematic evaluation, however, are not available. For instance, it is possible that *T. tridentatus*, which was not tested, is closer serologically to *Limulus* than *T. gigas* is. Further, since the Indo-Pacific serum supply was limited and only two rabbits were employed, one each, for the production of the antiserum to *T. gigas* and *C. rotundicauda*, a sharply defined serological relationship is impossible at this time.

The turbidimetric data are consistent, as the reciprocal tests show (Table 2). The greater reaction between *Limulus* serum and the antiserum of the two other species (46 and 59% or 53 and 67%), than that from the reciprocal reactions (35 and 31%), may indicate that the *Limulus* serum has one or more additional chemical components. The number of these components is suggested by several kinds of studies.

According to Allison & Cole (1940), hemocyanin is the only protein present in the serum of *Limulus polyphemus* after complete removal of the clot. They also noted that the clotting process, which involves the amoebocytes, does not



TEXT-FIG. 2. Two stages in the construction of a "serological yardstick" diagram show the relative distances between the three species: *Limulus polyphemus, Carcinoscorpius rotundicauda* and *Tachypleus gigas.*

remove any appreciable amount of hemocyanin from the serum. *Limulus* hemocyanin exists, within the pH range of 5.2-10.5, in four different and stable molecular species, but dissociation and a fifth component appear when the pH is less than 5.2 (Eriksson-Quensel & Svedberg, 1936). The electrophoretic pattern of *Limulus* hemocyanin reveals at least five components (Deutsch & McShan, 1949) and at least four prominent entire bands are revealed by the homologous test for *Limulus* in the agar-diffusion precipitin technique. The later technique reveals one and perhaps two major reaction bands that do not coalesce in any of the reciprocal reactions between *Limulus* and the two Indo-Pacific species. Reactions between only *Tachypleus* and *Carcinoscorpius* give entire but fewer bands.

Boyden (1943) found that the serological correspondence among marine crustaceans of the same genus averaged 46%. Later, in similar studies, Leone (1949, 1950) distinguished between species of the same genus within certain families of Crustacea. In his studies the magnitude of serological correspondence between crustaceans of the same genus was as low as 31%, but most values were above 70%, with some as high as 89% for closely related species. In our Limulidae study the heterologous reactions ranged from 30 to 67%. Although not directly comparable, the serological distance between species of the same genus of marine crustacea, when used as a "ruler" for comparison with the values among the Limulidae, suggests that the three species studied are congeneric.

Interpretation of the "distances" provided by the "serological ruler" is arbitrary, since "distance" depends upon a sliding scale which in turn is an expression of the specificity, *i.e.*, the discriminating capacity, of the antiserum which varies from antiserum to antiserum. Also, there are too few species and the present data are too scanty to establish more definite serological measurements of either species or genera within the Limulidae. Further, the best available reference for comparison is a distant taxonomic group comprised of many families, the Crustacea. The Limulidae is a very small taxon by comparison, but it is one that has roots in an old history, as measured by the geologic age of the Xiphosura, and one that reflects a conservative evolution.

If we consider that the present serological findings coincide with the current sub-Limulidae taxonomic categories given in the beginning of this report, then the serological distance between species, genera and families is indeed narrow.

The author wishes to express his gratitude and sincere thanks to the men most closely associated with the inception and progress of his studies on the Limulidae. Dr. Thurlow C. Nelson, Rutgers—The State University of New Jersey, counseled the writer in the formative years of the research. Dr. Harry A. Charipper, New York University, was largely responsible for the culminating stages of the dissertation preparation. Dr. Alfred C. Redfield, Woods Hole Oceanographic Institution, and Dr. William C. Cole, Rutgers—The State University of New Jersey, were instrumental in aiding the study through counsel and research grants from their respective institutions.

SUMMARY

From the turbidimetric measurements of precipitin reactions among the Limulidae sera and the rabbit-produced antisera it can be concluded that:

1. The precipitin reactions produced typical titration curves.

2. No demonstrable differences were found in the homologous reactions based on *Limulus* sera from different populations.

3. The sera of the two Indo-Pacific Limulidae tested, *Carcinoscorpius rotundicauda* and *Tachypleus gigas*, are readily distinguished from the sera obtained from different populations of *Limulus polyphemus*.

4. Serological correspondence is greatest between the two species of Tachypleinae, but *C. rotundicauda* may be serologically closer than *T. gigas* to *L. polyphemus*.

5. The three species of Limulidae are congeneric when compared to the same magnitude of serological correspondence among marine Crustacea.

6. The taxon including the Limulidae is limited in number of species, yet it exhibits a long geologic history and a conservative evolution. If the current classification is correct, wherein three of the four species are not congeneric, then the taxonomic categories of the extant species, genera and families are indeed narrow.

7. The results and other information reviewed in the discussion suggests that *Limulus* serum may have one or more components not present in the sera of the two Indo-Pacific species.

Data from agar-diffusion records of the precipitin reaction revealed:

1. Four major bands of reaction or components in *Limulus* homologous reactions.

2. A lack of correspondence for one and perhaps two major components between the Indo-Pacific species and *Limulus*.

3. An apparent complete correspondence between the two species of Tachypleinae.

The present study was limited by the lack of an adequate supply of sera from the Indo-Pacific species. More extensive serological studies would be of great value in interpreting the systematics and evolution of the Limulidae. These studies should include serum of the species *Tachypleus tridentatus* and employ, for example, the immunoelectrophoretic method (first used by Grabar & Williams, 1953, and emphasized as a requirement in systematic serology by Williams, 1956), among other techniques.

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EXPLANATION OF THE PLATE

Plate I

These retouched photographs of triangular serum-agar plates show the visible bands of precipitin reaction among the three species: *Limulus polyphemus, Carcinoscorpius rotundicauda* and *Tachypleus gigas.* The set of three letters around each triangle indicate the side from which the serum of each species (designated by CAPITAL LETTERS: L, C, and T) or antiserum to each species (lower case letters: l, c, and t) diffused. The eight sets of reactions, designated by the letters A through H, are described in the text.